Available online at www.sciencedirect.com
ScienceDirect
Virus Research 122 (2006) 171–174
Virus Research
www.elsevier.com/locate/virusres

Short communication

Synthesis of minus-strand copies of a viral transgene during viral infections of transgenic plants

Min Deng [1], William L. Schneider [2], Richard F. Allison *

Department of Plant Biology and Department of Plant Pathology, Plant Biology Building, Michigan State University, East Lansing, MI 48824-1312, USA

Received 15 February 2006; received in revised form 26 May 2006; accepted 31 May 2006
Available online 11 September 2006

Abstract

Viral transgenes designed to provide resistance to specific plant viruses frequently consist of the coat protein gene and a contiguous 3′ untranslated region (3′UTR) of viral origin. In many RNA viruses the viral 3′UTR establishes a recognition and initiation site for viral RNA replication. Thus the transgenic transcript may contain a functional virus replication site. Experiments were designed to determine if a challenging virus would recognize this replication site on a nuclear derived transcript and synthesize the complementary RNA. These data demonstrate that upon infection by a virus that recognizes the viral replication site, a full-length complement of the transgenic transcript is produced. In these experiments the replication complex of *Brome Mosaic bromovirus* recognized the transgenic transcript derived from a *Cowpea Chlorotic Mottle bromovirus* transgene. The resulting RNA may contribute to RNA recombination events.

Keywords: RNA recombination; Bromovirus

Viral protein mediated resistance is an effective means of limiting viral infections in numerous plant species (Fitchen and Beachy, 1993). While various viral transgene constructs including either the movement or replicase gene have provided virus resistance, most documented examples and current applications of viral gene mediated resistance involve the coat protein gene. The majority of these coat protein gene constructs include not only the coat protein gene itself but also the 3′ untranslated region (3′UTR) that flanks the coat protein gene in many plant RNA viral genomes (Powell-Abel et al., 1986; Stark and Beachy, 1989; Zaccomer et al., 1993). The 3′UTR may stabilize transgenic transcripts in the cytoplasm in much the same way as the structural complexity of the natural 3′UTR provides stability to the genomic viral RNA. Beyond its role in stability, the 3′UTR maintains the binding site for the viral replication complex (Ahlquist et al., 1981; Sriskanda et al., 1996; Bachman et al., 1994) and provides the initiation site for complementary strand synthesis.

In previous studies, *cowpea chlorotic mottle bromovirus* (CCMV) was used to demonstrate that transgenic viral gene transcripts are available in the cytoplasm for recombination with a replicating virus (Greene and Allison, 1994). Subsequently recombination events involving a viral transgene were observed in numerous other plant viral systems (Adair and Kearney, 2000; Borja et al., 1999; Schoelz and Wintermantel, 1993, 1996; Varrelmann et al., 2000). In the initial CCMV experiments, transgenic transcripts included part of the coat protein gene and its complete 3′UTR. In subsequent experiments elimination of all or part of the 3′UTR from the transgenic construct either prohibited recombination or lowered recombinant virus formation beyond detection (Greene and Allison, 1996). The presence of the 3′ UTR may have contributed to recombination events involving the transgene in two ways. First the secondary structure of the 3′UTR may have extended the transcript's cytoplasmic half life and availability to the replication complex by providing resistance to RNAses. Second, the replication complex binding site

Abbreviations: UTR, untranslated region; wt, wild type

* Corresponding author. Tel.: +1 517 432 1548; fax: +1 517 353 1926.
E-mail addresses: mindeng@uclink.berkeley.edu (M. Deng), william.schneider@ars.usda.gov (W.L. Schneider), allison@msu.edu (R.F. Allison).
[1] Present address: Plant and Microbial Biology, University of California, Berkeley, Koshland Hall, Berkeley, CA 94720, United States.
[2] Permanent address: Foreign Disease and Weed Science, USDA-ARS, 1301 Ditto Avenue, Ft. Detrick, MD 21702-5023, United States.

0168-1702/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.virusres.2006.05.009

may have remained functional and available to the challenging virus. If a complement of the transcript were formed, both the transgenic transcript and its complementary copy may have been involved in the demonstrated recombination events. Such events could explain the rather frequent detection of recombinants in the previous CCMV laboratory experiments and foretell similar events in the field.

Studies with several potyviruses and cucumber mosaic virus (Teycheney et al., 2000) demonstrated that a viral transgene is recognized and copied by viral replication complexes. The following experiments were designed to determine if a complementary RNA copy of the CCMV viral gene transcript could be detected in infected transgenic tissue and to estimate the quantity of product synthesized.

Both Northern blot and RT-PCR analyses were used in attempts to detect the complementary copy of a CCMV viral transgene in transgenic plants infected with either wild type (wt) CCMV or *brome mosaic bromovirus* (BMV). While these tripartite bromoviruses share little sequence homology, they have one known common host (*Nicotiana benthamiana*, Domin) plus BMV has the capacity to replicate CCMV RNA (Allison et al., 1988).

Three sets of plant materials were used in this study: nontransgenic *N. benthamiana,* and two clonally propagated transgenic *N. benthamiana* lines referred to as 3–57 and Δ69. Lines 3–57 contained a 694 nucleotide CCMV transgene consisting of 451 3′ nucleotides of the coat protein gene and the complete contiguous 243 nucleotide 3′UTR (Greene and Allison, 1994). Transgenic line Δ69 was similar but lacked the 69 terminal 3′ UTR nucleotides. The transgenic fragment of the coat protein gene used in both transgenic lines was distinguished from a similar region of the wild type virus by changing three nucleotides near the 3′ end of the coat protein gene to create a unique *Not*I restriction site (Greene and Allison, 1996). Although transcripts from these constructs were detected in Northern blots derived from both transgenic lines, neither line demonstrated resistance to either BMV or CCMV. *N. benthamiana* plants were inoculated with leaf extracts derived from either BMV or CCMV infected plants that were originally inoculated with transcripts of established cDNA clones (Ahlquist et al., 1984; Allison et al., 1988).

To define a period when virus replication was most active throughout the plant following basal leaf virus inoculation, dig-labeled probes specific for the 3′ UTR of the genomic RNAs of BMV and CCMV, HE1 and RA518(+) respectively (Ahlquist et al., 1981; Allison et al., 1990), were used to probe dot blots of crude extracts derived from BMV or CCMV inoculated *N. benthamiana* plants. Hybridization indicated that by 14 days post inoculation (dpi), infections had spread to all leaves of 45-day old plants (data not shown).

As single-stranded positive sense RNA viruses, bromoviruses replicate in the cytoplasm, where genomic RNA is a template for complementary minus-sense RNA synthesis (Marsh et al., 1991a). Northern blot detection of minus strand copies of CCMV RNAs in total RNA extracted from non-transgenic CCMV inoculated plants was unsuccessful. As a template for the genomic plus-sense RNA, the accumulation of minus strand RNA is approximately 100-fold less than plus strand RNA (Marsh et al., 1991b). Some minus-sense RNA remains associated with plus-sense templates and a relatively stable double-stranded RNA (dsRNA) can be isolated readily. This double-stranded RNA is resistant to mild ribonuclease treatment under high salt conditions (Hardy et al., 1979) and was the target of further hybridization experiments.

The dsRNA was denatured and Northern blotted (Hardy et al., 1979; Sethna et al., 1989). In the resulting blots minus strand CCMV RNA was detected by a ^{32}P-labeled RNA probe which recognizes the complementary copy of the 3′ UTR of CCMV RNAs. Using this RNA-RNA hybridization system, we were able to routinely detect the minus-sense genomic CCMV RNAs from 0.5 to 1.0 g of infected nontransgenic *N. benthamiana* plant tissue at 14 dpi. Optimization of sensitivity enabled detection of 10 pg of denatured plasmid DNA containing the cDNA copy of CCMV RNA3 (data not shown).

In an attempt to establish whether a complementary copy of the transgene was synthesized during virus infection, transgenic lines 3–57 and Δ69 were inoculated with either BMV or CCMV. At 14 dpi, dsRNA from two grams of leaf tissue was gel separated, blotted and probed with CCMV probe RA 518(−) (Allison et al., 1990). Only minus strand genomic RNA was detected. No minus strand band equivalent in size to the transgene was observed.

The calibrated sensitivity of the hybridization probe used in these Northern blot assays indicated that if a minus strand copy of the transgene was synthesized during infection, its concentration was below the probe's sensitivity. Thus if a complementary copy of the transgene is synthesized, its concentration in dsRNA form must be well below that of genomic dsRNA and likely less than 10 pg/2 g of leaf tissue.

To better search for the complementary copies of the viral transgene, a more sensitive technique, RT-PCR, was used. In this procedure, total RNA from the above mentioned plant tissue was treated with RNase-free DNase I to remove the plant genomic DNA including the chromosomal copy of the viral transgene. A minus-strand CCMV RNA3 specific oligonucleotide (5′-AAGTGGATCCCCTCTTGTGCGGCTGC-3′) primed first-strand cDNA synthesis. DNA synthesis was completed using the primer 5′-ACTCCAAAGAGTTCTTCCG-3′, which anneals near the 5′ terminus of the Δ69 transcript. The predicted size of the RT-PCR fragment was 572 bp. As shown in Fig. 1, lanes 7, 10 and 13, a minus-strand RNA was amplified in all CCMV infected 3–57, Δ69, and nontransgenic plants. A band of the correct size was also present in the BMV infected 3–57 transgenic plants (Fig. 1, lane 12). This minus-sense RNA was not observed in any mock-inoculated transgenic plants, BMV infected Δ69 plants or non-transgenic plants (Fig. 1, lanes 5, 6, 8, 9 and 11).

To determine if the 572 bp RT-PCR products originated from transcripts of the transgene, PCR products were digested with *Not*I. The *Not*I site characterizes only the transgene and a mutated form of CCMV, AG1, and is not present in the wild-type CCMV inoculum. The RT-PCR product derived from a plant inoculated with AG1 was completely cleaved by the *Not*I restriction enzyme Fig. 2, lane 3, but that of wild type CCMV was not cleaved (Fig. 2, lane 5). The RT-PCR product of CCMV

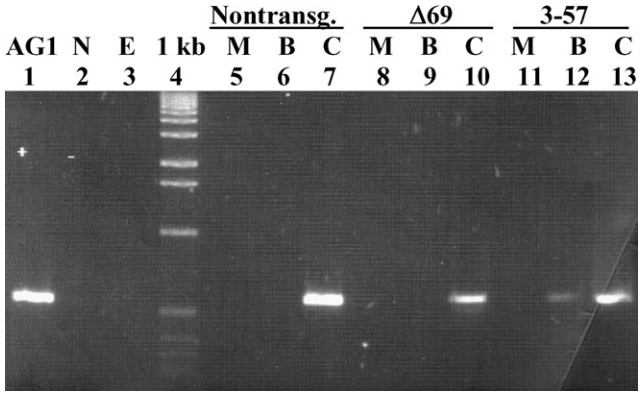

Fig. 1. Agarose gel electrophoresis with 572 bp RT-PCR amplified fragment of negative sense CCMV RNA3 derived from total RNA from nontransgenic and transgenic lines. Plants were inoculated with either BMV (B) or CCMV (C). Lanes: 1. CCMV control AG1, 2. PCR negative control, 3. empty, 4. 1 kb size marker (Gibco BRL). Lanes 5–7, 8–10 and 11–13 were derived from nontransgenic, transgenic Δ69 and 3–57 plants respectively. M indicated mock-inoculated plants.

infected Δ69 plants was not cleaved by *Not*I (Fig. 2, lane 7). This indicates that the minus-strand CCMV RNA amplified in CCMV infected Δ69 transgenic plants were from the wild type CCMV. The RT-PCR product of CCMV infected 3–57 plants was partially cleaved by *Not*I (Fig. 2, lane 11), indicating that the minus-sense CCMV RNA3, which was PCR amplified, was of two origins: most of the RNA was from wild type CCMV inoculum while some was from the transgenic RNA transcript. For BMV infected 3–57 transgenic plants, the RT-PCR product was completely cleaved by *Not*I. Note that no CCMV PCR fragment is present in the BMV inoculated Δ69 plants (Fig. 1, lane 9). Thus the RT-PCR amplified fragments in lanes 8 and 9 of Fig. 2 were derived solely from the viral transgene that had been converted to a minus strand during BMV replication. This indicates that both the CCMV and BMV replication complexes recognized the replication site on the CCMV transgenic transcript that contained the complete CCMV 3′ UTR and synthesized a complementary copy of the transcript.

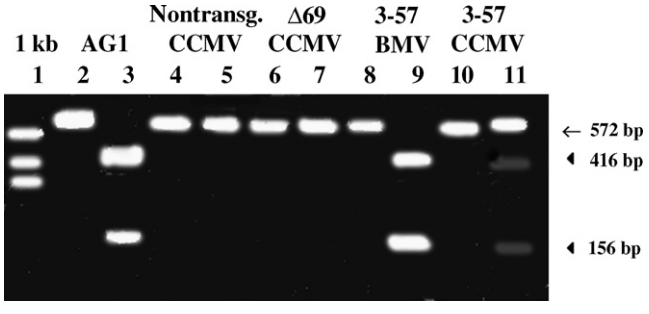

Fig. 2. Agarose gel comparing non-digested and *Not*I digested RT-PCR products amplified from total RNA extracted from virus infected plant tissue. Lane 1 contains the 1 kb size marker. Control lanes 2 and 3 are PCR products derived from CCMV AG1, which contains the *Not*I site. Lanes 4 and 5 represent CCMV infected nontransgenic plants, lanes 6 and 7 are CCMV infected Δ69, lanes 8 and 9 are BMV infected 3–57 and lanes 10 and 11 are from CCMV infected 3–57. PCR products in lanes 3, 5, 7, 9 and 11 were treated with *Not*I while products in even numbered lanes were not digested. The arrow denotes undigested fragments while pointers indicate *Not*I digestion fragments.

This study complements a previous report that describes similar viral transgene replication of several potyviruses and CMV in tobacco (Teycheney et al., 2000). This work examines viral transgene replication in *N. benthamiana* using two additional bromoviruses, BMV and CCMV. It supports and expands the previous report by suggesting the quantity of the transgene complement is below 10 pg in a 2 g leaf sample. Additionally, this work supports the possibility that either the transgenic transcript itself or its complement may be involved in the recombination events noted previously (Greene and Allison, 1994).

Collectively these studies indicate that inclusion of the replication complex binding site in a transgenic construct may lead to the synthesis of a complement of the transgenic transcript. This unanticipated transcript may also contribute in recombination events involving RNA viruses that may challenge the transgenic plant. With both forms of the transgenic transcript available, RNA recombination could occur during either positive or negative strand synthesis. The absence of the transgenic transcript's complement in the Δ69 plants emphasizes the requirement for a complete 3′UTR for replication involvement. Thus, in the interest of prohibiting synthesis of a complement of the transgenic transcript and thereby inhibiting its possible involvement in RNA recombination events, the natural 3′ UTR should either be modified by deletion or point mutations. Alternatively, the 3′ UTR could be replaced by a suitable stabilizing nucleotide sequence.

Acknowledgments

This work was supported by award number 2001-52106-11223 Biotechnology Risk Assessment Research Grants Program/USDA.

References

Adair, T.L., Kearney, C.M., 2000. Recombination between a 3-kilobase tobacco mosaic virus transgene and a homologous viral construct in the restoration of viral and nonviral genes. Arch. Virol. 145, 1867–1883.

Ahlquist, P., Dasgupta, R., Kaesberg, P., 1981. Near identity of 3′ RNA secondary structure in bromoviruses and cucumber mosaic viruses. Cell 23, 183–189.

Ahlquist, P., French, R., Janda, M., Loesch-Fries, S.L., 1984. Multicomponent RNA plant virus infection derived from cloned viral cDNA. Proc. Natl. Acad. Sci. U.S.A. 81, 7066–7070.

Allison, R.F., Janda, M., Ahlquist, P., 1988. Infectious in vitro transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. J. Virol. 62, 3581–3588.

Allison, R.F., Thompson, C., Ahlquist, P., 1990. Regeneration of a functional RNA virus genome by recombination between deletion mutants and requirement for cowpea chlorotic mottle virus 3a and coat genes for systemic infection. Proc. Natl. Acad. Sci. U.S.A. 87, 1820–1824.

Bachman, E.J., Scott, S.W., Xin, G., Vance, V.B., 1994. The complete nucleotide sequence of prune dwarf ilarvirus RNA 3: implication for coat protein activation of genome replication in ilarviruses. Virology 201, 127–131.

Borja, M., Rubio, T., Scholthof, H.B., Jackson, A.O., 1999. Restoration of wild-type virus by double recombination of tombusvirus mutants with a host transgene. Mol. Plant–Micorb. Interact. 12, 153–162.

Fitchen, J.H., Beachy, R.N., 1993. Genetically engineered protection against viruses in transgenic plants. Ann. Rev. Microbiol. 47, 739–763.

Greene, A.E., Allison, R.F., 1994. Recombination between viral RNA and transgenic plant transcripts. Science 263, 1423–1425.

Greene, A.E., Allison, R.F., 1996. Deletions in the 3′ untranslated region of cowpea chlorotic mottle virus transgene reduce recovery of recombinant viruses in transgenic plants. Virology 225, 231–234.

Hardy, S.F., German, T.L., Loesch-Fries, L.S., Hall, T.C., 1979. Highly active template-specific RNA-dependent RNA polymerase from barley leaves infected with brome mosaic virus. Proc. Natl. Acad. Sci. U.S.A. 76, 4956–4960.

Marsh, L.E., Huntley, C.C., Pogue, G.P., Connell, J.P., Hall, T.C., 1991a. Regulation of (+):(−)-strand asymmetry in replication of brome mosaic virus RNA. Virology 182, 76–83.

Marsh, L.E., Pogue, G.P., Huntley, C.C., Hall, T.C., 1991b. Insight to replication strategies and evolution of (+) strand RNA viruses provided by brome mosaic virus. Oxford Surv. Plant Mol. Cell Biol. 7, 297–334.

Powell-Abel, P.A., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T., Beachy, R.N., 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232, 738–743.

Sethna, P.B., Hung, S.L., Brian, D.A., 1989. Coronavirus subgenomic minus-strand RNAs and the potential for mRNA replicons. Proc. Natl. Acad. Sci. U.S.A. 86, 5626–5630.

Schoelz, J.E., Wintermantel, W.M., 1993. Expansion of viral host range through complementation and recombination in transgenic plants. Plant Cell 5, 1669–1679.

Schoelz, J.E., Wintermantel, W.M., 1996. Isolation of recombinant viruses between cauliflower mosaic virus and a viral gene in transgenic plants under conditions of moderate selection pressure. Virology 223, 156–164.

Sriskanda, V.S., Pruss, G., Ge, X., Vance, V.B., 1996. An eight-nucleotide sequence in the potato virus X 3′ untranslated region is required for both host protein binding and viral multiplication. J. Virol. 70, 5266–5271.

Stark, D.M., Beachy, R.N., 1989. Protection against potyvirus infection in transgenic plants: evidence for broad-spectrum resistance. Biotechnology 7, 1257–1262.

Teycheney, P., Aaziz, R., Dinant, S., Salanki, K., Tourneur, C., Balazs, E., Jacquemond, M., Tepfer, M., 2000. Synthesis of (−)-strand RNA from the 3′ untranslated region of plant viral genomes expressed in transgenic plants upon infection with related viruses. J. Gen. Virol. 81, 1121–1126.

Varrelmann, M., Palkovics, L., Maiss, E., 2000. Transgenic or plant expression vector mediated recombination of plum pox virus. J. Virol. 74, 7462–7469.

Zaccomer, B., Cellier, R., Boyer, J.C., Haenni, A.L., Tepfer, M., 1993. Transgenic plants that express genes including the 3′ untranslated region of the turnip yellow mosaic virus (TYMV) genome are partially protected against TYMV infection. Gene 136, 87–94.